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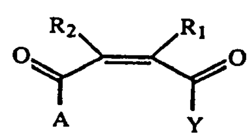
(54) Title: A METHOD OF INACTIVATING HUMAN IMMUNODEFICIENCY VIRUS



II

(57) Abstract

The present invention relates to a method of inactivating human immunodeficiency virus (HIV) which comprises administering to a HIV infected host a therapeutic dosage of a compound of the general formula A-X-Y, wherein A is an antibiotic residue selected from the group consisting of anthracyclines, mitomycins, ellipticines and derivatives thereof; X is a coupling agent selected from the group consisting of glutaraldehyde and maleic anhydride derivatives which gives a compound of general formula (II), R_1 and R_2 are each independently members selected from the group consisting of: hydrogen atom; phenyl; phenyl substituted by at least one member selected from the group consisting of hydroxy, halogen, lower alkyl, lower alkoxy or nitro; C_{1-4} alkyl; C_{1-4} alkoxy; and C_{1-6} carboxyalkyl; with the proviso that R_1 and R_2 cannot be simultaneously a hydrogen atom, and when one of R_1 or R_2 is a hydrogen atom, the other one cannot be $-CH_2COOH$; and Y is a protein residue linked to X via the amino residue of a ε-lysine or -SH residue of a cysteine present therein, whereby the Y residue reacts directly with the HIV or HIV infected cells or the Y residue maintains a desired level of antibiotic in the blood circulation for a prolonged period of time while decreasing the drug's toxicity.

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TITLE OF THE INVENTION

A method of inactivating human immunodeficiency virus.

BACKGROUND OF THE INVENTION

The acquired immunodeficiency syndrome (AIDS) is one of the most devastating diseases of the immune system. The disease is the end result of the destruction of the immune system by a lymphotropic and lymphocytopathic retrovirus, designated human immunodeficiency virus (HIV). Although HIV infection may lead to the acquired immunodeficiency syndrome, infection by this virus leads to a diverse set of clinical outcomes. Indeed the majority of patients who acquire this virus appear to remain asymptomatic without developing marked immunologic abnormalities. A smaller yet substantial proportion of the group appears to develop stable lymphadenopathy designated AIDS related complex (ARC). A relatively small proportion of patients with ARC actually develop AIDS, although the risk rises with time. AIDS, is the end result of severe immunodeficiency with the development of either opportunistic infections such as Pneumocystis carinii pneumonia or neoplasms, such as Kaposi's sarcoma or aggressive non-Hodgkin's lymphoma. The reasons for this diversity of outcomes of the acquisition of this virus remain largely unknown. In addition, it seems that individuals within the various risk groups may exhibit marked immunologic abnormalities as a result of HIV infection but without evidence of clinical illness.

Development of Therapies for AIDS:

As a general principle, a therapeutic agent against a pathogen must either kill the pathogen or stop it from growing without significantly harming the patient. Retroviruses such as HIV are particularly difficult to treat because they are an elusive target. They are not only intracellular pathogens but their life cycle is characterized by their ability to survive and grow as persistent low grade infections in CD4 positive cells. Such cells can act as a mean to spread the disease to other tissues such as the central nervous system. where they become less accessible to therapeutic agents because of the blood brain barrier. Also secondary diseases associated with AIDS may make the disease even more difficult to treat.

Virtually no drug is free of undesirable side effects because host genome or proteins cannot be easily differentiated from the virus genomes or products. Thus, a fair balance must be achieved between the damage to the pathogen and damage to the host, the so-called "therapeutic index". Most of the current strategies for the development of treatment for HIV are focused on drugs that may attack different stages of the life cycle of HIV such as binding, uncoating, reverse transcription, translation, protein modification, assembly and budding. The drug AZT (3'-azido,3'-dideoxythymidine) is only partially successful in treating AIDS patients which may be related to the fact that monocytes phosphorylate AZT poorly which is required for this drug to be effective. HIV replication in monocytes appears to be more prolonged and less cytolytic than in lymphocytes.

The frequency of infected blood monocytes from HIV seropositive patients has been reported as very low by 'in situ' hybridization (Harper, M.E. et al., PNAS, 1986, vol. 83, p. 772). However, Crowe, S. et al., ICACC N.Y. N.Y., 1988, Abstract have reported that the frequency of HIV postive monocytes is higher (3-33% of blood monocytes) and this number increases in short term cultures to 12-45%. The development of drugs that target monocytes/macrophages use a variety of endogenous enzymatic systems to destroy the virus. One such system that can be explored is the generation of superoxide anions and hydroxyl radicals. Monocytes produce such products to destroy and eliminate foreign material that they phagocytose. Monocytes under suitable experimental conditions can be activated to produce sustained hydroxyl radicals (Britigan, B.E., J. Exp. Med., 1988, vol. 168, p. 2367). These radicals and anions are employed by the monocytes to kill bacteria and viruses (Babior, B.M., New Engl. J. Med., 1978, vol. 298, pp. 659 & 671). The mechanism of destruction is through protein, lipid oxidation and scission of DNA and RNA.

The European Patent Application number 0, 294, 763 (Kuramochi Tsuneo, published on December 14, 1988) discloses the use of anthracycline antibiotics as anti-retroviral agent for the treatment of AIDS and adult T-cell leukemia (ATL). Nevertheless, the anthracycline antibiotics were tested on feline leukemia virus (FeLV) infected cells. The anthracycline antibiotics turned out to be effective against the FeLV but were never tested against any of the human retroviruses. Also, it was never taken into consideration

that AIDS patients do suffer from a defective immune system and the toxicity of the anthracycline antibiotics, at the doses employed, would probably kill them.

An article of anthracycline's effect on Kaposi's sarcoma in Cancer Treatment Reports (Abraham Chachoua et al., 1987, vol. 71, nos 7-8, July-August), concluded that anthracyclines were not effective against this neoplastic disease. Kaposi's sarcoma is a neoplastic disease associated with AIDS. It is a secondary disease due to the presence of HIV but is not an infectious disease.They also state that the toxicity is mainly hematologic causing leukopenia and thrombocytopenia. Non-hematologic toxicity consisted of nausea, vomiting, diarrhea and cardiac dysfunction.

It was demonstrated that anthracyclines have an antiviral utility but because of their toxicities, they cannot be administered to AIDS patients '<u>per se</u>'.

In general, drug products are formulated for administration by a variety of routes, including oral, buccal, sublingual, rectal, parenteral, topical, and inhalation. The physicochemical properties of drugs, their formulations, and the routes of administration are important in their absorption. Absorption is the process of drug movement from the site of application toward the systemic circulation, whereas bioavailability is the rate at which and the extent to which the active moiety (drug or metabolite) enters the general circulation, thereby permitting access to the site of action.

Absorption from Parenteral Sites

The subcutaneous injection of drugs bypasses the skin barrier, but the drug must penetrate into the capillaries. The rate of entry into capillaries is usually determined for lipid-soluble drugs by their oil/water partition coefficients and for lipid-insoluble drugs by their molecular size. The rate of capillary blood flow is also a major factor in the rate of absorption. Thus, the injection site and the blood flow to it can markedly influence a drug's absorption rate.

Prolonged-Release Dosage Forms

Prolonged-(controlled) release dosage forms are designed to reduce the frequency of dosing and to maintain more uniform plasma drug concentrations, thus providing a more uniform pharmacologic effect. Additionally, greater patient convenience may improve compliance with the therapeutic regimen.

It would be highly desirable to be able to target an anthracycline or a mitomycin against HIV in order to specifically inactivate the virus and decrease deleterious effects of the drug on the patient.

It would also be desirable to have drugs that will preferentially result in the production of superoxide anions and hydroxyl radicals which could work on either one of the following:

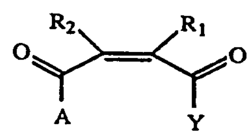
1) HIV infectivity;
2) Inactivation of HIV in monocytes.

SUMMARY OF THE INVENTION

Surprisingly and in accordance with the present invention, there is provided a method of inactivating HIV which comprises administering to a HIV infected host a therapeutic dosage of a compound of general formula A-X-Y, wherein:

A is an antibiotic residue selected from the group consisting of anthracyclines, mitomycins, ellipticines and derivatives thereof;

X is a coupling agent selected from the group consisting of glutaraldehyde and maleic anhydride derivatives which gives a compound of the general formula II



II

R_1 and R_2 are each independently members selected from the group consisting of:

(1) hydrogen atom;

(2) phenyl;

(3) phenyl substituted by at least one member selected from the group consisting of hydroxy, halogen, lower alkyl, lower alkoxy or nitro;

(4) C_{1-4} alkyl;

(5) C_{1-4} alkoxy; and

(6) C_{1-6} carboxyalkyl;

with the proviso that R_1 and R_2 cannot be simultaneously a hydrogen atom, and when one of R_1 or R_2 is a hydrogen atom, the other one cannot be $-CH_2COOH$; and

Y is a protein residue linked to X via the amino residue of a ε-lysine or -SH residue of a cysteine present therein, whereby the Y residue reacts with HIV or HIV infected cells or the Y residue maintains a desired level of antibiotic in the blood circulation for a prolonged period of time while decreasing the drug's toxicity.

The protein residue (Y) is used to target the drug to HIV present in an infected host while decreasing the drug's toxicity.

The compound of the present invention is able to generate oxygen radicals ($0_2^{\cdot -}$ and $OH^{\cdot -}$) close to HIV virions and has the following advantages:

I- this compound easily penetrates the cell walls;

II- this compound is reducible by a NADH or NADPH dependant diaphorase in order to make sure the radicals are specifically generated inside the cell;

III- the reductive activation and generation of oxygen radicals is performed at physiological pH and temperature without light or apparatus.

Other advantages of the present invention will be readily illustrated by referring to the following description.

IN THE DRAWINGS

Figure 1 is a representation of plasmatic concentration-time relationship after a single dose of a hypothetical anti-viral agent administered by itself or conjugated to a protein.

Figure 2 is a graph which demonstrates the effect of daunorubicin (Cerubidine®) on HIV replication.

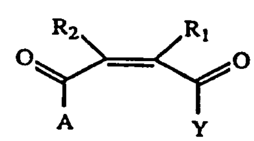
Figure 3 is a graph which demonstrates the effect of daunorubicin conjugated to maleylated human serum albumin in HIV replication.

DETAILED DESCRIPTION OF THE INVENTION

The method of the present invention of inactivating HIV comprises administering to a HIV infected host a therapeutic dosage of a compound of the general formula A-X-Y, wherein:

A is an antibiotic residue selected from the group consisting of anthracyclines, mitomycins, ellipticines and derivatives thereof;

X is a coupling agent selected from the group consisting of glutaraldehyde and maleic anhydride derivatives which gives a compound of the general formula II



II

R_1 and R_2 are each independently members selected from the group consisting of:

(1) hydrogen atom;

(2) phenyl;

(3) phenyl substituted by at least one member selected from the group consisting of hydroxy, halogen, lower alkyl, lower alkoxy or nitro;

(4) C_{1-4} alkyl;

(5) C_{1-4} alkoxy; and

(6) C_{1-6} carboxyalkyl;

with the proviso that R_1 and R_2 cannot be simultaneously a hydrogen atom, and when one of R_1 or R_2 is a hydrogen atom, the other one cannot be $-CH_2COOH$; and

Y is a protein residue linked to X via the amino residue of a ε-lysine or a -SH residue of a cysteine present therein, whereby the Y residue reacts with HIV or HIV infected cells or the Y residue maintains a desired level of antibiotic A in the blood circulation for a prolonged period of time while decreasing the drug's toxicity.

The bioavailability of any drug can be in any of the three following zones. First, the non-therapeutic zone is the zone wherein the drug plasmatic concentration is not high enough to show any pharmacological activity. Secondly, the therapeutic zone is the zone wherein the drug plasmatic concentration is high enough to show a desired pharmaceutical activity. Thirdly, the zone of toxicity is the zone at which the plasmatic drug concentration is too high and confers toxicity to its host. There is observed, in the zone of toxicity, various undesirable effects begining by minor side effects (ex. nausea,

vomitting ...), followed by toxic effects (ex. anemia, alopecia, cardiotoxicity ...) and eventually death of the patient.

It is shown in Figure 1 that the non-conjugated anti-viral agent remains for a short period of time in the therapeutic zone. It also has to be given at such a high concentration that it remains in the zone of toxicity for a certain period of time.

On the other hand, the conjugated anti-viral agent while taking more time before any therapeutic activity can be seen, never reaches the zone of toxicity while remaining in the therapeutic zone for a prolonged period of time.

In the case of AIDS patients, it is a great advantage to have a drug which does not show any zone of toxicity since these patients suffer from a defective immune system and are generally very weak.

The compound of the present invention has the following advantages:

1) the drug has reduced toxicity due to the administration of lower drug doses;

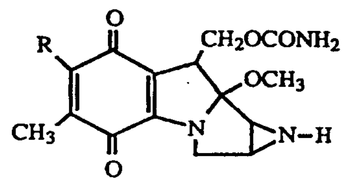
2) the drug has a longer half-life;

3) the drug efficacy is increased; and

4) the drug is also more specific and is able to distinguish virus infected versus non infected cells.

As an example of anthracycline, there may be mentioned daunorubicin, doxorubicin, epirubicin and derivatives thereof.

As an example of mitomycin derivatives, there may be mentioned the one of general formula III described by Iyengar B.S. et al. (J. Med. Chem., 1986, 29, 1760; J. Med. Chem., 1983, 26, 16) listed below;



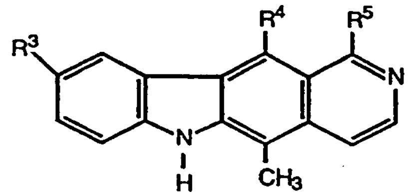
III

wherein,

R is selected from the group consisting of :

$-NH(CH_2)_2NH_2$;
$-NH(CH_2)_3NH_2$;
$-NH(CH_2)_2SH$;
$-NH(CH_2)_2OH$;
$-NH(CH_2)_2O(CH_2)_2OH$;
$-NH(CH_2)_2O(CH_2)_2NH_2$;
$-NH(CH_2NH_2)_2$;
$-NH(CH_2)_4NH(=NH)CNH_2$;
$-NHCH_2(OH)CHCH_2NH_2$; and
$-NHCH_2(OH)CHCH_2OH$.

As an example of ellipticine, there may be mentioned the one of general formula IV,



IV

wherein,

R^3 is selected from the group consisting of:

(a) hydrogen;
(b) NH_2;
(c) SH; and
(d) hydroxy;

R^4 and R^5 are selected from the group consisting of:

(a) hydrogen; and
(b) lower alkyl;

with the proviso that R^4 and R^5 cannot be simultaneously a lower alkyl.

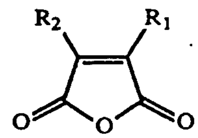
Different combinations of R^3, R^4 and R^5 are listed in Table I.

Table I

R^3	R^4	R^5
H	H	H
H	CH_3	H
H	H	CH_3
NH_2	CH_3	H
SH	CH_3	H
OH	CH_3	H
OH	H	CH_3
SH	H	CH_3
NH_2	H	CH_3

The compounds of formula II are generally prepared as follows:

a- by reacting an anti-viral agent with a compound of the general formula V



V

wherein R_1 and R_2 are as defined previously;

b- by purifying the A-conjugation agent complex by extraction with dichloromethane, and/or other suitable organic solvents in aqueous acidic solution; and

c- reacting the dissolved A-conjugation agent complex in phosphate buffer solution with a desired protein carrier (Y) and isolating the conjugated compounds from the reaction mixture.

As an example of suitable proteins, there may be used albumin, albumin derivatives, red blood cells, red blood cells derivatives, blood platelets, antibodies against viral products and antibodies against viral receptors.

Cell Lines

The cell lines were only used as targets to show that the conjugate can be directed to the desired sites. As an example of suitable cell lines there may be mentioned HUT 78 (TIB161) and U937 (CRL 1593). These cell lines are readily available from the American Type Culture Collection under the numbers shown in brackets.

All cell lines are routinely cultured in RPMI-1640® medium supplemented with 10% fetal bovine serum and 100 ug per mL of streptomycin and 100 ug per mL of penicillin.

As targets to show that the conjugate can be directed to the desired sites, there may be also used blood samples obtained from infected patients.

Presence or absence of HIV virus

In order to evaluate the efficiency of the method of the present invention, the reverse transcriptase (RT) assay is used and other methods for determining the presence or absence of HIV could have been used.

The enzymatic activity of reverse transcriptase is proportional to the concentration of HIV present in a cell or supernatant.

The method to determine if infectious virus is present is to determine reverse transcriptase activity. This enzyme is absolutely necessary for the virus to replicate. The RT of the virus is very specific and by providing the correct substrate, it determines the presence or absence of the virus. The level of the enzymatic activity can also provide an indication of infection. The assay essentially is the '<u>in vitro</u>' synthesis of deoxyribonucleic acid (DNA). One of the nucleic acids is labelled radioactively which can be introduced in the DNA. After the reaction has proceeded for a certain period of time,

the nucleic acids are separated from DNA. This method is widely used to determine the presence of HIV.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its scope.

Example I

Preparation of a daunorubicin-X-M-HSA conjugate when X is glutaraldehyde.

I- Preparation of maleylated human serum albumin (M-HSA)

Human serum albumin (30mg) (M.wt.=69.000, 4.3×10^{7} moles) is dissolved at room temperature in 2mL of phosphate buffer solution (PBS) pH 7.4. Maleic anhydride (680µg) (7×10^{6} ,16 molar excess, in dioxan) is added to the solution and the mixture is gently stirred at room temperature for 1 hour. It is purified by gel filtration on Sephadex G-25® column. The maleylated human serum albumin is collected in the void volume of the column.

II- Conjugation with glutaraldehyde of daunorubicin to maleylated human serum albumin

Glutaraldehyde (100µL of 0.1% solution) is added to 1mL of maleylated human serum albumin (M-HSA, 10mg) and daunorubicin (DNR, 200µg). The mixture is incubated at 37°C for 30 minutes and purified by gel filtration on Sephadex G-25®.

There is obtained the daunorubicin-glutaraldehyde-M-HSA conjugate.

Example II

Preparation of a daunorubicin-X-antibody conjugate, when X is a maleic anhydride derivative.

I- Activation of daunorubicin with maleic anhydride derivatives (as described in a separate patent pending)

Daunorubicin (DNR) is activated with cis aconitic anhydride according to a modification of the procedure reported by Shen and Ryser (Biochem. Biophys. Res. Comm. (1981), 102 :1048). Thirty mg of daunorubicin sold by Rhône-Poulenc under the name Cerubidine® dissolved in 10 mL of phosphate buffer 0,1M at pH 7.5 are extracted by 4X 10 mL of dichloromethane. The organic phase is evaporated under reduced pressure, without heating. The red residue, 5 mg of daunorubicin is dissolved in 10 mL of $NaHC0_3$ 7% and 16 mg of cis aconitic anhydride are added portionwise over 1 hour (4 x 4mg). Thirty minutes after the final addition of cis aconitic anhydride, the mixture is extensively extracted with dichloromethane in order to remove all unreacted daunomycin. The pH of the solution is lowered around 3 by addition of HCl 0.1M and the mixture extracted with ethyl acetate. The organic phases are dried over Na_2SO_4 and evaporated in vacuo. The residue is dissolved in DMSO and the yield is determined by spectrophotometry (495 mm) E = 196.

TLC on silica gel ($CHCl_3$: MeOH: H_20) (80:30:4)

R_fDNR - 0.3 R_f CIS aconityl DNR 0.2

II- Conjugation to antibody

To a buffered solution (pH 7.5-8.0) of the antibody is added cis aconityl daunorubicin and 25 molar excess of EDCI (1-(3-

dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride). The mixture is stirred at 37°C) for 1 hour and chromatographied on Sephadex G-25® (PD-10 columns) with phosphate buffer.

There is obtained the daunorubicin-cis aconityl-antibody conjugate.

Example III

Drug evaluation of daunorubicin on HIV infected cells.

I- The preparation of tRNA from yeast was prepared as follows:

The stock tRNA was dissolved in 10 mM Tris/HCl, 0.1 M NaCl and 1 mM EDTA. The stock tRNA was diluted 10x in the same buffer for use.

II- Solutions to be used for harvesting the precipitated DNA

a) 5% Trichloroacetic acid(TCA)+0.01% sodium pyrophosphate
b) 10% Trichloroacetic acid + 0.01% sodium pyrophosphate
c) 70% Ethanol

Note: TCA to precipitate DNA
Na pyrophosphate to prevent absorption of [^{3}H]TTP

Method:

1) Take the required volume of [^{3}H]TTP (Amersham, TRK-576, 1 mCi in 50% ethanol) and evaporate the solvent under a gentle flow of nitrogen in a fume hood.

2) Redissolve to the appropriate volume with 10mM Tris/HCL, pH 7.9.

3) Add the other reagents to make a stock of 2x reaction buffer.

4) Place 50 ul of sample(s) to be tested into the wells of a flat bottomed microtiter plate.

5) Add 50ul of 2x reaction buffer to each of these wells.

6) Cover the plates and incubate at 37°C for 22 hours.

7) After incubation, add 10 ul of tRNA to each well, followed by 100 ul of cold 10% TCA.

8) After precipitation, the plates are harvested with a semi-automatic cell harvester (Skatron®).

a) A filter sheet (grade 25®, Schleicher and Schuell) is prewet with 70% ethanol for 30 seconds, using a row of empty wells on the plate.

b) Place the nozzles of the harvester into the row to be harvested, and put through automatic wash cycle of: (i) 5% TCA:

ii) 10% TCAs 30 seconds for each wash.

c) Use the air button to air dry the glass fibre filter.

d) Move the filter along so that the next row of samples can be collected.

e) Repeat steps A, B, C, D, until all samples have been harvested.

f) Remove the glass fibre filter sheet from the harvester, and let dry completely in the air.

g) Transfer the disks containing the precipitable DNA to plastic scintillation vials, and add 3mL of scintillation fluid to each vial.

h) Count vials in a beta-scintillation counter.

i) The results will be expressed as counts per minute (CPM).

III- Inactivation of HIV virus

All samples are inactivated before testing. The inactivation is performed with 4'-aminoethyltrioxalen hydrochloride (AMT sold by Lee Biomolecular Research Inc., San Diego, California) and an ultra violet light trans-illuminator (Spectroline® model TC-365, Fisher Scientific, Ottawa, Ontario). The

AMT is reconstituted in 50% ethanol at 1 ug/mL. The sample is aliquoted in eppendorf tubes and for every 100 ul of supernatant, 10 ul of AMT is added to the sample. The samples are layed in the trans-illuminator and irradiated for 5 minutes. An additional 10 ul of AMT is added to the sample and the samples are irradiated for a further 5 minutes. The samples are now inactivated.

IV: Method for drug evaluation (non-conjugated daunorubicin) on HIV infected cells.

U937 cells are obtained and cell viability is assessed by trypan blue dye exclusion. Viability is assessed as 100% and the concentration of cells is 5.2 X 10^5 cells/mL at the time of harvest. The cells are either not infected or infected with HIV (RT activity=1 X 10^6 CPM/50 ul). A total of 1mL of infectious virus is added to 10^7 U937 cells. An equivalent amount of cells is treated with media only. Both populations are kept separate and incubated for 1 hr at 37°C. Both populations are resuspended in culture media (the cells are not washed) and cultured for an additional 24 hrs at 37°C and 5% CO_2. The cells are washed four times to remove as much as possible unbound HIV and are resuspended in culture medium at a density of 1 X 10^7 cells/mL.

The cells infected with HIV are subdivided into 2 groups. The first received no antibiotic at all (test 1). The second received 1, 5, 10, 25 or 50 ng/mL of daunorubicin (cerubicine®, DNR) (test 2, 3, 4, 5, 6). The cells that are not infected with HIV are also subdivided into 2 groups. The first received no drug (control 1) and the second received 1, 5, 10, 25, and 25 ng/mL of daunorubicin (cerubicine®) (control 2, 3, 4, 5, 6). The cells are cultured for an additional 3 days at

37°C and 5% CO. The number of replicates that is used for each of test 1 and controls 1 is 24 whereas tests 2 to 6 and control 2 to 6 are performed 12 times each.

The following test and assays are performed:

1) visual inspection with the reverse phase microscope;
2) cell number enumeration;
3) cell viability; and
4) reverse transcriptase activity.

<u>Results</u>

Visual Inspection

At the end of culture period, the cells are viewed under the reverse phase microscope to determine if visible microscopic changes could be observed. All cells in test 1 to 5 and control 1 to 5 are near confluency and are not affected by the addition of Cerubidine® and or HIV. However the cultures having the highest concentration of drug (50 ng/mL) are affected. Giant cells are seen but not enumerated. Both HIV and noninfected cultures are equally affected.

Cell number enumeration

Cell number enumeration and viability is performed by mixing each culture (the cells do not attach to the plate) and taking an aliquot and adding an equal volume of trypan blue dye. The cells are treated with AMT to deactivate the virus and counted on the microscope. The results are given in Table II.

TABLE II

Control	cells/mL(10^6)	% viable	ng/mL (DNR)
1	1.4	100	0
2	1.5	100	1
3	1.4	100	10
4	.79	90	25
5	.59	96	50
Test	**cells/mL(10^6)**	**% viable**	**ng/mL (DNR)**
1	1.4	100	0
2	1.3	100	1
3	1.4	100	10
4	.94	98	25
5	1.0	90	50

Effect of daunorubicin on HIV replication

At the end of the culture period,the RT assay is performed. The cultures are harvested, spun at 1300 rpm, and the supernatants are inactivated with AMT, aliquoted and frozen at -20°C until RT activity is performed. AMT does not have an effect on reverse transcriptase activity. The RT activity of the cultures is shown in figure 2.

Discussion:

This experiment clearly demonstrates that HIV is affected by anthracycline drugs. The results clearly demonstrate that the drug affects cell growth at the 50 ng/mL, less at 25 ng/mL and essentially not at all at lower doses, but with minimal effect on viability (Table II). However at all drug doses, cell growth occurs. Cultures are seeded at 0.2 x 10^6 cells per ml on day 0 and at the end of the experiment, all cultures demonstrate cell growth from 0.59 to 1.4 x 10^6 cells (i.e. 2.5 to 7 times more cells than originally seeded). The viability is not 100% but

ranges from 90 to 100%. Therefore, the drug at the doses employed does not kill the cells but the drug does retard their growth.

The reduction in reverse transcriptase activity is dramatic (figure 2). This result does not mean that we have completely eliminated the virus from these cells. It may mean that we have reduced virus load which is very important in an effective treatment of AIDS patients. The current therapy for AIDS is AZT, which is employed to reduce virus load. AZT is not very effective in AIDS patients since it is poorly phosphorylated in monocytes. The monocytes have been postulated to be the reservoir for HIV. In the present invention the monocytes have been targeted for therapy and we are devising drugs which can employ the machinery of the cell to be effective thus providing a specific site for action. The effect on virus levels is overwhelming. At 50 ng/mL the virus is completely inactivated. A reduction in virus load as measured by reverse transcriptase activity is observed in cultures with concentrations of drug as low as 10 ng/ml. No virus replication is observed at the 50 ng/ml concentration even in the presence of cell growth which usually stimulates virus production. In addition, it must be emphasized that monocytes and the cell line U937 are not killed with virus infection as is seen with T cells. Therefore, we have not lost preferentially cells infected with HIV. We have reduced dramatically virus concentrations in our cultures.

Example IV

Drug evaluation of daunorubicin conjugated to protein carrier on HIV infected cells.

Daunorubicin is linked to maleylated human serum albumin (M-HSA) by the glutaraldehyde method. Maleylated albumin is employed since cells of the monocyte lineage have a specific receptor for this compound (Hamilton et al., J. of Immunol., 1987, vol. 138, p. 4063). Thus, monocytes can be specifically targeted by employing these substances. An experiment is performed with D-M-HSA and M-HSA. Sham treatment of M-HSA on HIV replication is assessed also. The experiment is performed substantially as described in Example III except that the drugs are daunorubicin-maleylated human serum albumin (D-M-HSA) and M-HSA. The same concentrations of daunorubicin are employed 1, 5, 10, 25, 50 ng/mL respectively. The concentration of M-HSA in the cultures is identical to the concentration of HSA in the D-M-HSA preparation. As in Example III step IV both virus infected and non-infected cells are tested to determine non specific toxicity of the drug. Two other controls are also included in the experiment. The first control has no virus and no treatment whereas the second control has HIV, but no treatment with any drug (D-M-HSA or M-HSA).

Results

Cell number enumeration

Cell number enumeration and viability is performed by mixing each culture (the cells do not attached to the plate) and taking an aliquot and adding an equal volume of trypan blue dye. The cells are treated with AMT to deactivate the virus and counted on the microscope. The results are given in Table III.

TABLE III

NON-INFECTED CELLS			
M-HSA			
Control	cells/mL(10^6)	% viable	ng/mL (DNR)
1	1.0	100	0
2	1.16	100	1
3	1.22	100	5
4	1.24	100	10
5	1.22	100	25
6	1.35	100	50
D-M-HSA			
Test	cells/mL(10^6)	% viable	ng/mL (DNR)
1	1.32	100	0
2	1.25	100	1
3	1.12	100	5
4	0.96	100	10
5	0.58	100	25
6	0.42	100	50
HIV INFECTED CELLS			
M-HSA			
Control	cells/mL(10^6)	% viable	ng/mL (DNR)
7	1.0	100	0
8	1.16	100	1
9	1.22	100	5
10	1.24	100	10
11	1.22	100	25
12	1.35	100	50
D-M-HSA			
Test	cells/mL(10^6)	% viable	ng/mL (DNR)
7	1.28	100	0
8	1.16	100	1
9	1.00	100	5
10	0.88	100	10
11	0.34	100	25
12	0.32	100	50

Effect of daunorubicin (Cerubidine®) on HIV

At the end of the culture period,the RT assay is performed. The cultures are harvested, spun at 1300 rpm, and the supernatants are inactivated with AMT, aliquoted and frozen at -20°C until RT activity is performed. AMT does not have an effect on reverse transcriptase activity. The RT activity of the cultures is shown in figure 3.

Discussion:

The effect of different concentrations of D-M-HSA on cell growth and viability is nearly identical to what is observed with the drug only. M-HSA does not affect cell growth and viability, thus the carrier is not toxic and killing the cells (control 2 to 6 and 8-12, Table III). The D-M-HSA does retard cell growth beginning at 10 ng/ml which is not observed with 10 ng/ml when drug alone, without carrier, is employed (Table II). Thus, the drug D-M-HSA seems to be more effective in reducing virus infectivity than drug alone. The increase effectiveness of the drug is probably due to the specific receptors on monocytes for M-HSA (Hamilton et al., J. of Immunol., 1987, vol. 138, p. 4063).

The drug D-M-HSA seems to be more effective than drug only since the cell number after culture (50 ng/ml) is much lower than is observed with drug only at the same drug concentration (1.0 vs 0.32). The homing of the drug via specific receptor on the cells probably increases the effectiveness of the drug.

The increase viability (Table III) is also observed when the drug D-M-HSA is employed. At 50 ng/ml, 100% cell viability is observed when using D-M-HSA (Table III), whereas only 90% is observed when the drug only is used (Table II).

The reverse transcriptase activity of cultures treated with D-M-HSA is also decreased and possibly completely eliminated. The drug D-M-HSA seems to be more effective since at 10 ng/ml, the reverse transcriptase activity is completely abrogated whereas the reverse transcriptase activity of cultures with drug alone is not completely eliminated.

In summary, daunorubicin linked to a specific carrier can inactivate HIV without seriously affecting cell growth and viability. The form of this drug is more effective than daunorubicin without carrier.

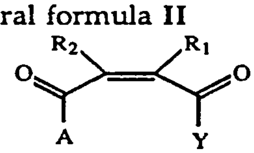
While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known and customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

WE CLAIM:

1. A method of inactivating HIV which comprises administering to a HIV infected host a therapeutic dosage of a compound of the general formula A-X-Y, wherein:

A is an antibiotic residue selected from the group consisting of anthracyclines, mitomycins, ellipticines and derivatives thereof;

X is a coupling agent selected from the group consisting of glutaraldehyde and maleic anhydride derivatives which gives a compound of the general formula II



II ,

R_1 and R_2 are each independently members selected from the group consisting of:

(1) hydrogen atom;

(2) phenyl;

(3) phenyl substituted by at least one member selected from the group consisting of hydroxy, halogen, lower alkyl, lower alkoxy or nitro;

(4) C_{1-4} alkyl;

(5) C_{1-4} alkoxy; and

(6) C_{1-6} carboxyalkyl;

with the proviso that R_1 and R_2 cannot be simultaneously a hydrogen atom, and when one of R_1 or R_2 is a hydrogen atom, the other one cannot be $-CH_2COOH$; and

Y is a protein residue linked to X via the amino residue of a ε-lysine or -SH residue of a cysteine present therein, whereby the Y residue reacts with HIV or HIV infected cells or the Y residue maintains a desired level of antibiotic in the blood circulation for a prolonged period of time while decreasing the drug's toxicity.

2. The method according to Claim 1, wherein A as an anthracycline is selected from the group consisting of daunorubicin, doxorubicin, epirubicin and derivatives thereof suitable for conjugation to the Y residue

3. The method according to Claim 1, wherein A is a mitomycin and derivatives thereof suitable for conjugation to the Y residue.

4. The method according to Claim 1, wherein A is a ellipticine and derivatives thereof suitable for conjugation to the Y residue.

5. The method according to Claim 1, wherein Y is selected from the group consisting of albumin, albumin derivatives, red blood cells, red blood cells derivatives, blood platelets, antibodies raised against viral products and antibodies raised against viral receptors.

1/2

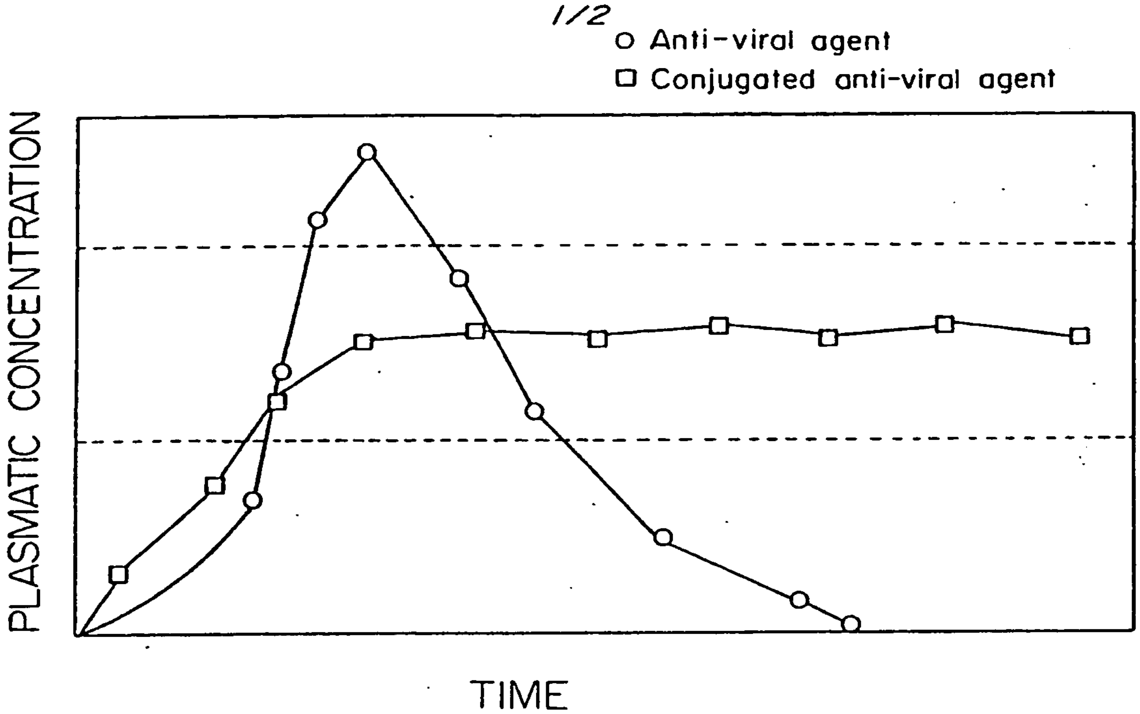

o Anti-viral agent
□ Conjugated anti-viral agent
PLASMATIC CONCENTRATION
TIME

FIG. 1

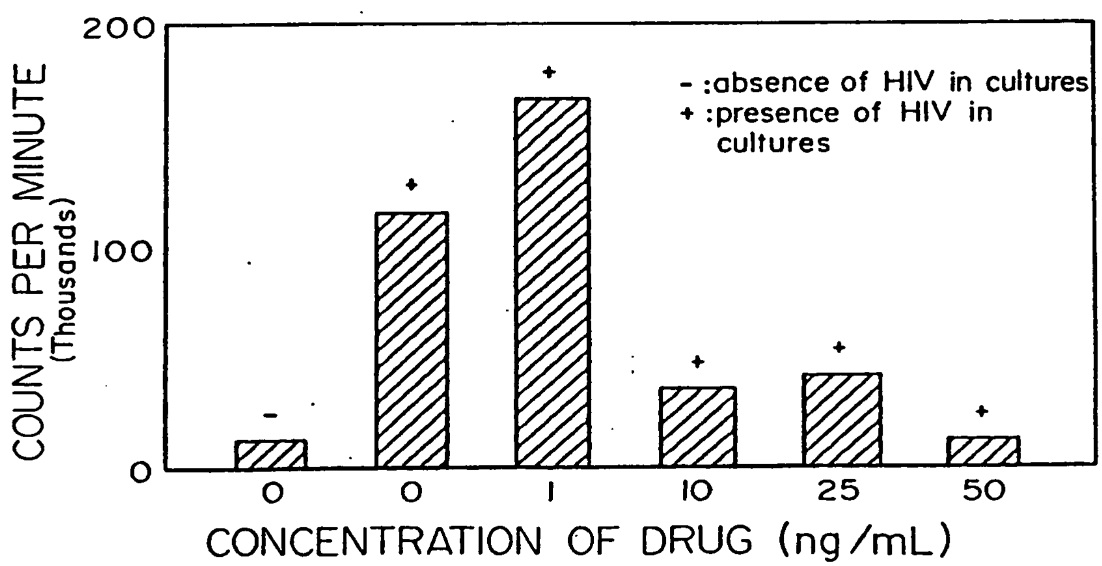

200
100
0
COUNTS PER MINUTE (Thousands)
- :absence of HIV in cultures
+ :presence of HIV in cultures
-
+
+
+
+
+
0
0
1
10
25
50
CONCENTRATION OF DRUG (ng/mL)

FIG. 2

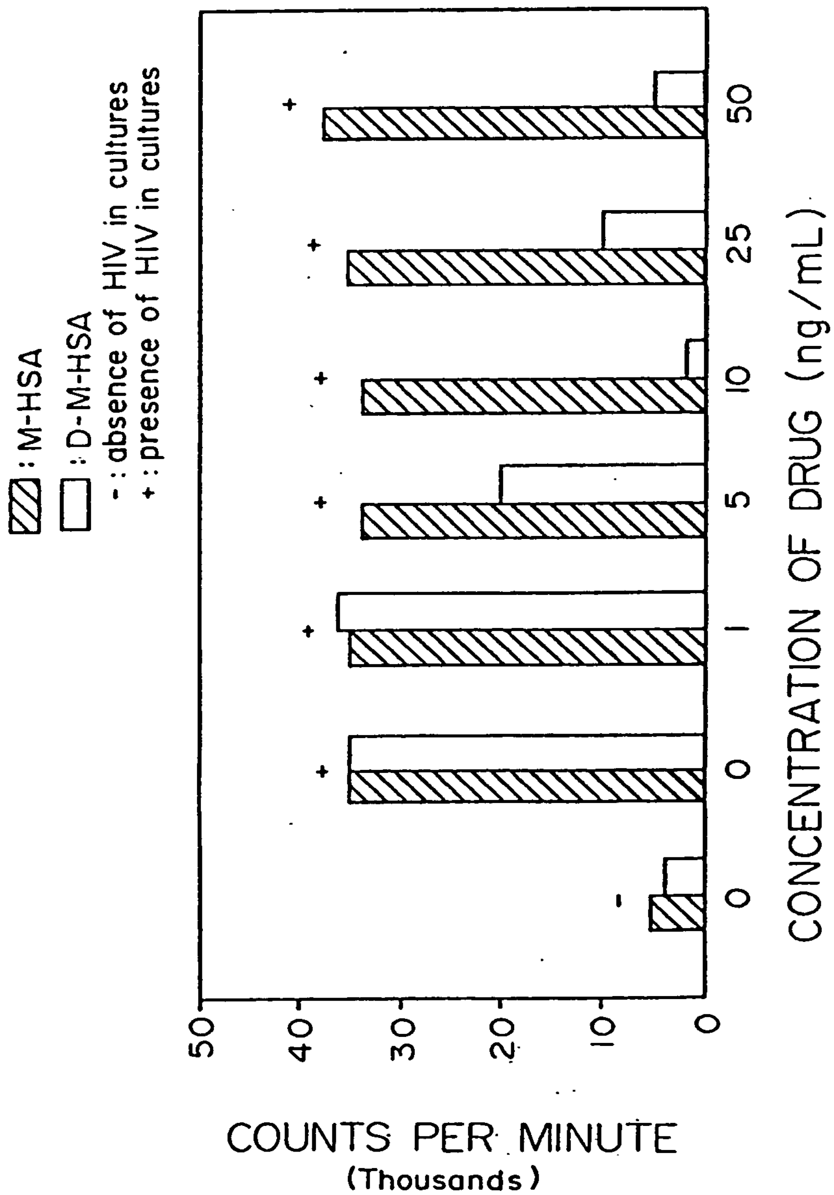

: M-HSA
: D-M-HSA
- : absence of HIV in cultures
+ : presence of HIV in cultures
0
10
20
30
40
50
COUNTS PER MINUTE
(Thousands)
0
0
1
5
10
25
50
CONCENTRATION OF DRUG (ng/mL)

FIG. 3

INTERNATIONAL SEARCH REPORT

International Application No PCT/CA 90/00423

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: A 61 K 47/48, C 12 N 7/06

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	A 61 K, C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Makromol. Chem., Suppl. 2, 1979, (Basel, CH), M. Wilchek: "Affinity therapy and polymer bound drugs", pages 207-214, see page 209; page 211, paragraph 2; page 213 --	1-5
Y	The Journal of Antibiotics, vol. 40, no. 3, March 1987, (Tokyo, JP), H. Nakashima et al.: "Inhibition by doxorubicin of human immuno-deficiency virus (HIV) infection and replication in vitro", pages 396-399 see the whole article --	1-5
P,Y	Chemical Abstracts, vol. 113, no. 17, October 1990, (Columbus, Ohio, US), A. Ahmad et al.: "Daunorubicin coupled to monoclonal antibodies via a cis-aconitic anhydride linker: Biochemical ./.	1-5

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
7th February 1991	15. 03. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Natalie Weinberg

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (Continued from Form PCT/ISA/206 Annex)

Category*	Citation of Document with indication, where appropriate, of the relevant passages	Relevant to Claim N°
	and cytotoxic properties revisited", see page 28, abstract 144961p & Anticancer Res. 1990, 10(3), 837-43	
	--	
A	EP, A, 0294763 (MEIJI SEIKA KAISHA LTD) 14 December 1988 see the whole document (cited in the application)	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. CA 9000423 SA 42088

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 11/03/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0294763	14-12-88	JP-A- 63310830	19-12-88
		US-A- 4908438	13-03-90

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82